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Suppressor of T-cell receptor signalling 1 and 2 differentially regulate endocytosis and signalling of receptor tyrosine kinases

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Abstract **Suppressor of T-cell receptor signalling 1 and 2 (Sts-1 and 2) negatively regulate the endocytosis of receptor tyrosine kinases. The UBA domain of Sts-2 and SH3-dependent Cbl-binding are required for this function. Sts-1 and -2 also possess a PGM domain, which was recently reported to exhibit tyrosine phosphatase activity. Here, we demonstrate that the PGM of Sts-1, but not of Sts-2, dephosphorylates the EGFR at multiple tyrosines thereby terminating its signalling and endocytosis. In contrast to Sts-2 the UBA of Sts-1 did not contribute significantly to receptor stabilization. Thus, although Sts-1 and Sts-2 are structurally highly homologous and both inhibit ligand-induced EGFR degradation, their mechanisms of action differ significantly. As a consequence, Sts-1-containing receptor complexes are inactive, whereas Sts-2-containing complexes are signalling competent.**

Keywords: Sts-1; Sts-2; Phosphatase; UBA; PGM endocytosis; Receptor; Tyrosine phosphorylation

1. Introduction

Receptor tyrosine kinase (RTK) signalling promotes growth, survival, proliferation, migration and differentiation in mammalian cells in response to extracellular stimuli [1]. Ligand-binding initiates the dimerization of the cognate receptors and the activation of their kinase domains. Consequent autophosphorylation on multiple tyrosines within the cytosolic tail of the receptors creates docking sites for a variety of adaptor and effector proteins containing phosphotyrosine-binding domains. They in turn trigger the intracellular propagation of the extracellular signal that finally results in the activation of target genes in the nucleus [1]. Duration and amplitude of signalling are tightly controlled in order to avoid aberrant activation that can lead to uncontrolled cell growth, migration, transformation or proliferation and is frequently associated with developmental defects or tumorigenesis [2].

Dephosphorylation of the receptor by phosphatases is the most rapid and efficient way for transient signal termination [3]. Permanent inactivation of the EGFR is achieved by its internalization and sorting into lysosomes where it is degraded by lysosomal proteases [2]. To this end the receptor needs to be ubiquitinated by the E3-ligase Cbl, which is recruited to the autophosphorylation site Tyr1045 of the receptor and via the adaptor protein Grb2 that binds to phosphorylated Tyr1068. The Ub-moieties attached to the receptor are recognized by a number of endocytic adaptor proteins that mediate the transport along the endolysosomal route [4].

Suppressors of T-cell receptor signalling (Sts) 1 and 2 are Cbl-interacting proteins that have been found to protect EGFR and PDFGR from lysosomal degradation [5,6]. While Sts-1 is ubiquitously expressed in mammalian organisms Sts-2 expression is confined to the hematopoietic lineage [7,8]. Sts-1 and 2 share about 40% sequence identity. Both proteins possess a N-terminal Ub-associated (UBA) domain, a SH3 domain, that mediates binding to the proline-rich motif of Cbl, and a C-terminal phosphoglycerate mutase (PGM) domain that mediates dimerization of Sts-1/2 and is otherwise of unknown function [5,6]. The UBA domain of Sts-2 was found to be required for the stabilizing effect on EGFR. It has been proposed that Sts-1 and -2 bind to the ubiquitinated receptor thereby competing with other UDB-containing endocytic adaptor proteins such as the epsins or Eps15 that would sort the ubiquitinated receptor to the lysosomes [5]. Accordingly, overexpression of Sts-2 led to prolonged EGFR signalling and cellular transformation [5]. In addition, the UBA domain of Sts-1 and -2 mediate E2-mediated self-monoubiquitination that results in the inactivation of their Ub-binding ability by inducing intramolecular UBA/Ub interactions [9,10]. Sts-1 and -2 are also critical regulators of T-cell activation. Mice lacking Sts-1 and -2 are hyperresponsive to T-cell stimulation and show hyperactivation of signaling proteins downstream of the T-cell receptor (TCR) including ZAP70 [7].

Here, we show that in contrast to Sts-2 the UBA domain of Sts-1 is not involved in stabilization of activated EGFR. However, the PGM domain of Sts-1, but not Sts-2, dephosphorylates the EGFR and allows its escape from Cbl-mediated downregulation. Though closely related in domain architecture and amino acid sequence Sts-1 and Sts-2 exploit different strategies of regulating EGFR signalling.

2. Experimental procedures

2.1. Reagents, cells, plasmids and antibodies

Constructs for FLAG-tagged Sts-1 and Sts-2 have been described recently [9]. Phosphataseinactive mutants of Sts-1 and Sts-2 have been generated by site-directed mutagenesis of

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residues Arg390 and His391 of Sts-1 and Arg364 and His365 of Sts-2 to alanines.

Antibodies anti-FLAG (M2 and M5) antibodies from Sigma and anti-GST antibodies from Santa Cruz. For immunoprecipitation from HeLa and HEK 293T-cells the following antibodies were used: EGFR: anti-EGFR 528 (Santa Cruz); FLAG-tagged proteins: M5 (Sigma); Cbl: anti-Cbl RF. Phosphotyrosine-specific EGFR antibodies were purchased from Cell Signalling and anti-phosphotyrosine (pY99), anti-Erk2 and anti-phospho-Erk2 from Santa Cruz.

For overexpression experiments, HEK 293T-cells were transfected using Lipofectamine Reagent® (Invitrogen) and Hela cells using Effectene (Quiagen) according to the manufacturer's instructions.

Human EGF was purchased from Peprotech.

2.2. Immunoprecipitation

HEK 293T or HeLa cells were transfected with the indicated constructs, lysed 24–48 posttransfection for 10 min on ice in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 25 mM NaF, 10 μM $ZnCl_2$, pH 7.5) containing protease inhibitors (aprotinin, leupeptin, and PMSF) and *ortho*-vanadate. Cell lysates were collected, centrifuged for 15 min (13 000 rpm) to remove

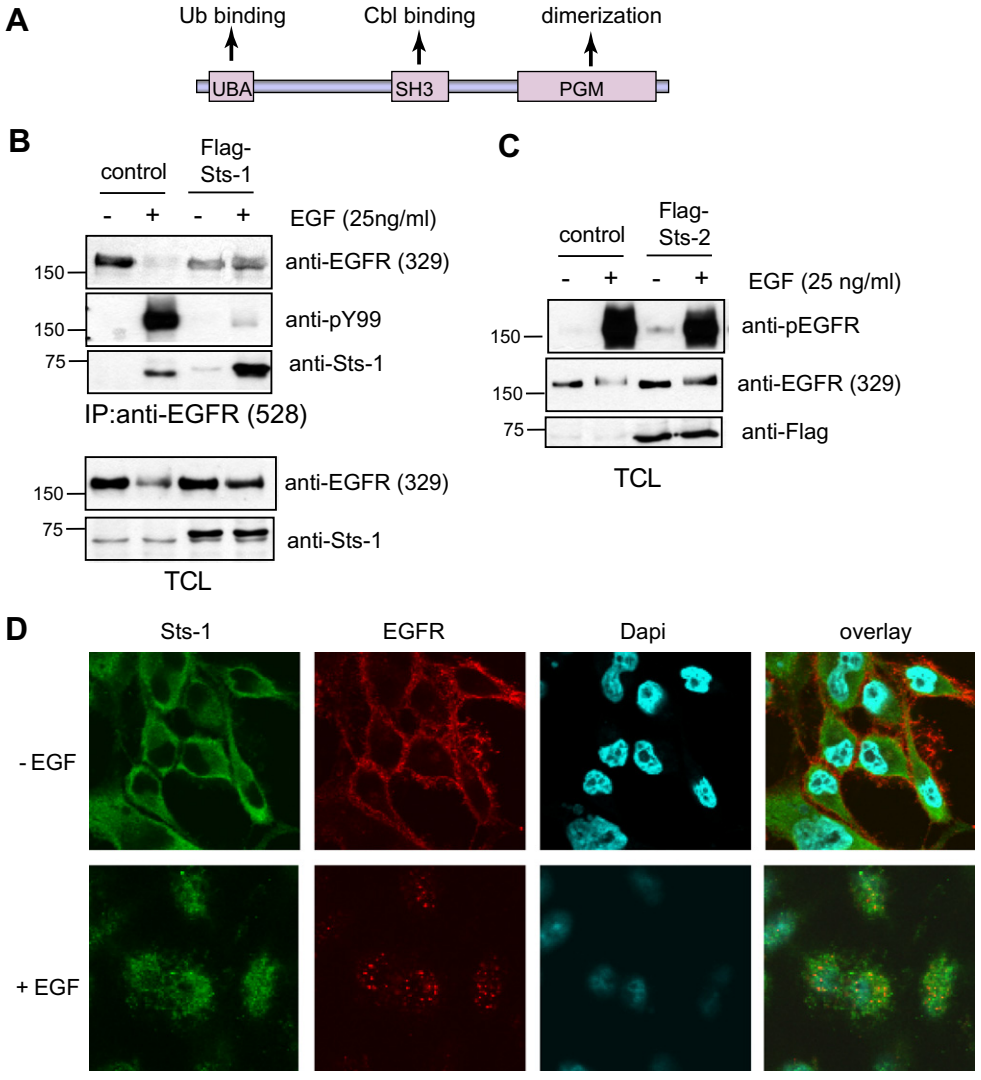

Fig. 1. Sts-1/2 are recruited to activated EGFR complexes. (A) Sts proteins possess an N-terminal UBA domain that binds the conserved hydrophobic patch of ubiquitin, a SH3 domain that mediates the association with the proline-rich region in Cbl and a C-terminal phosphoglycerate mutase domain. (B) Endogenous Sts-1 and overexpressed Flag-Sts-1 are recruited into activated EGFR complexes, dephosphorylate and stabilize EGFR. HeLa cells expressing the indicated constructs, were starved over night and stimulated for 5 min with 25 ng/ml EGF. Cell lysates were subjected to immunoprecipitation using an EGFR-specific antibody (anti-EGFR 528) and analyzed by immunoblotting using the indicated antibodies. (C) FLAG-Sts-2 is recruited to activated EGFR complexes and stabilize EGFR after EGF stimulation but does not dephosphorylate the receptor. HeLa cells expressing the indicated constructs, were starved over night and stimulated for 5 min with 25 ng/ml EGF. Cell lysates were subjected to immunoprecipitation using an EGFR-specific antibody (anti-EGFR 528) and analyzed by immunoblotting. (D) In unstimulated HeLa cells (upper panel) endogenous Sts-1 shows a diffuse cytosolic distribution and endogenous EGFR is localized at the cell surface membrane. After stimulation with 25 ng/ml EGF for 5 min EGFR and Sts-1 colocalize in vesicles (lower panel). The presented image shows a section through the upper part of the cells close to the cell membranes.

the Triton X-100-insoluble fraction and incubated with the indicated antibody and Immunosorb Protein A beads (Medicago, Sweden) for >3 h at +4 °C. After incubation, the sepharose matrix was washed 3 times with lysis buffer and bound proteins were eluted by boiling the samples for 5 min with Laemmli buffer containing 5% β-mercaptoethanol.

2.3. Confocal microscopy

Hela cells were seeded onto coverslips and transfected with FLAG-Sts-1 or FLAG-Sts-1 RH/AA or left untransfected. After serum starvation for 8 h, the cells were stimulated for 5 min with 25 ng/ml EGF. Cells were fixed with 4% PFA, permeabilized with digitonin and stained for EGF receptor with a monoclonal mouse antibody 528 (Santa Cruz) and for FLAG-Sts-1 using a polyclonal anti-FLAG antibody (Sigma, 1:300). Secondary antibodies conjugated with fluorochromes (anti-rabbit-FITC and anti-mouse-Cy3, Jackson Immunoresearch) were used to visualize the primary antibodies. Images were prepared using a Zeiss 510 Meta confocal microscope.

2.4. Internalization assay

Hela cells were transfected either FLAG tagged Sts-1, Sts-1 RH/AA, Sts-1 UBA* or empty vector (control) and GFP as a transfection control in 10 cm cell culture dishes. After 24 h cells were split into 12 well dishes and starved overnight in serum-free medium. The next day cycloheximide (20 μg/ml) was added to the cells 2 h before they were left unstimulated or were incubated with EGF (25 ng/ml) for 5, 15 and 30 min at 37 °C. After stimulation cells were harvested using accutase (PAA), blocked for 15 min in 4% BSA/PBS on ice and incubated with PE-coupled anti-EGFR antibody (BD biosciences) for 30 min to detect cellsurface EGFR. For each sample, 10,000 GFP-positive cells were analyzed using the Epics XL flow cytometer (Beckman-Coulter). Mean fluorescence

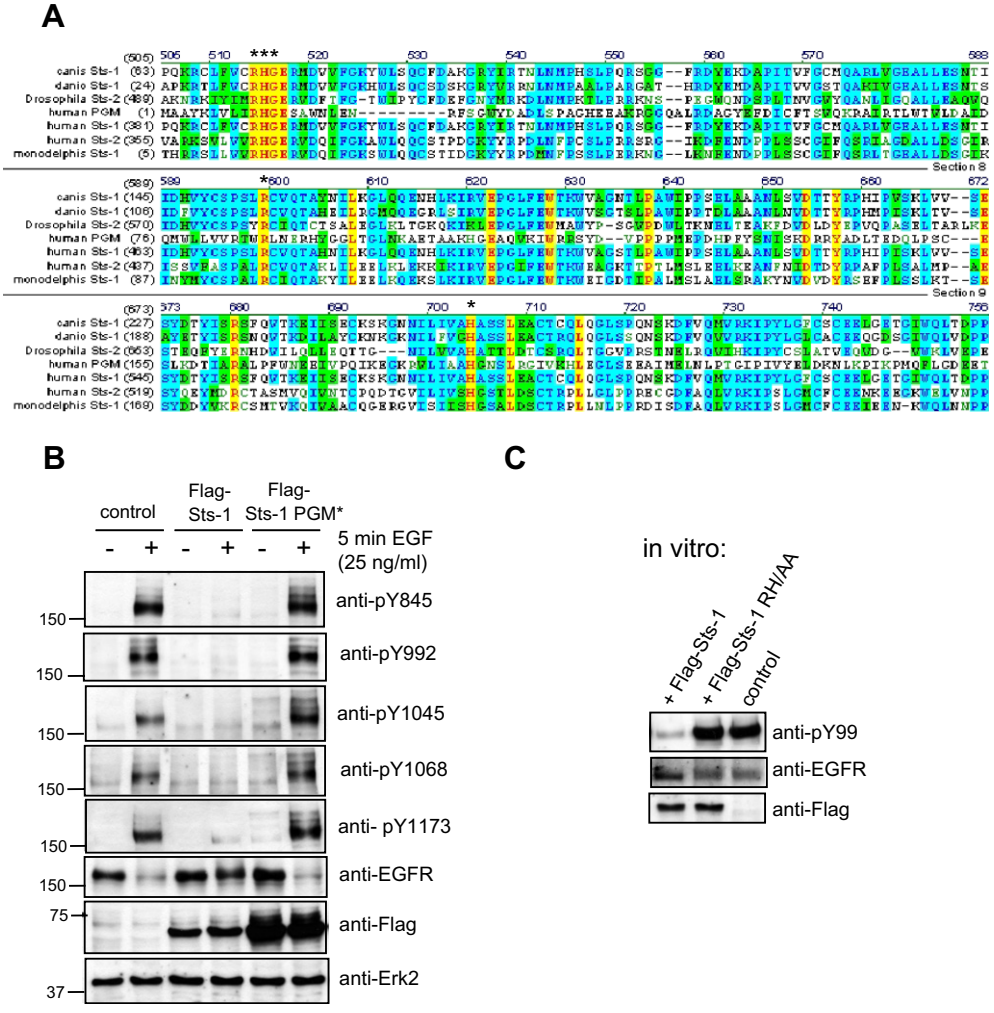

Fig. 2. Sts-1 dephosphorylates endogenous EGFR at multiple tyrosines. (A) Amino acid sequence alignment of Sts-1/2 PGM domains and human PGM. Highly conserved residues are highlighted in yellow and the PGM/AcP signature motif is labelled with asterisks. (B) Sts-1 overexpression leads to dephosphorylation of multiple tyrosins and mutation of the conserved Arg390 and His391 abolishes this effect. HeLa cells expressing the indicated constructs were starved overnight and stimulated with 25 ng/ml EGF for 5 min. Cell lysates were analyzed by SDS–PAGE and immunoblotting using phosphotyrosine-specific antibodies. (C) EGFR is a direct target of Flag-Sts-1. Phosphorylated EGFR was precipitated from HeLa cells stimulated for 5 min with EGF using anti-EGFR (528) antibody and washed with RIPA buffer. FLAG-Sts-1 and FLAG-Sts-1 RH/AA were overexpressed in HEK 293T-cells and immunoprecipitated using anti-FLAG (M5) antibody. After stringent washing with RIPA buffer the proteins were eluted from the beads with FLAG peptide, added to the EGFR beads and incubated for 30 min at 37 °C. Phosphorylation of EGFR was assessed by SDS–PAGE and immunoblotting using an phosphotyrosine-specific antibody (pY99).

intensity of each sample was calculated using Expo 32 ADC software. Equal expression of the transfected proteins was checked by immunoblotting.

3. Results and discussion

In order to characterize the differential mechanisms underlying Sts-1- and Sts-2-dependent inhibition of EGFR endocytosis we analyzed the behaviour of endogenous EGFR in the absence and presence Sts-1 (Fig. 1A) and Sts-2 (Fig. 1B). Upon stimulation of HeLa cells with 25 ng/ml EGF overexpressed FLAG-Sts-1, FLAG-Sts-2 and endogenous Sts-1 (HeLa cells do not contain Sts-2) were recruited into complexes with endogenous EGFR. Additional transfection of Cbl led to increased recruitment of Sts-1/2 ([5] and data not shown). Moreover, overexpression of Sts-1 and Sts-2 led to a marked inhibition in the receptor downregulation after stimulation with EGF. These results confirm previous observations that Sts-1 and -2 are recruited via Cbl into activated EGFR complexes and inhibit EGFR downregulation [5]. However, when we analyzed the phosphorylation status of EGFR after stimulation with EGF we detected a dramatic loss of tyrosine phosphorylation of EGFR upon overexpression of Sts-1 (Fig. 1A). This effect was not observed in case of Sts-2 (Fig. 1B). We additionally analysed endogenous Sts-1 using confocal microscopy. In unstimulated HeLa cells Sts-1 showed a diffuse cytosolic localization (Fig. 1D, upper panel). Treatment of the cells with 25 ng/ml EGF lead to the redistribution into vesicular structures that also contained EGFR (lower panel).

The C-terminal domain of Sts-1 and Sts-2 resembles members of the phosphoglycerate mutase/acidic phosphatase family which act as phosphatases or phosphotransferases [11]. An alignment of the amino acid sequences of Sts-1/2 from different species and human PGM reveals that the overall identity is low, but the signature motif of the PGM/AcP family, comprising two histidines (H) and two arginines (R), are highly conserved in the Sts-1-proteins (Fig. 2A, involved residues are labelled with asterisks). We mutated Arg390 and His391 in Sts-1 (Sts-1 RH/AA) and Arg364 and His365 in Sts-2 (Sts-2 RH/AA). After expression in HEK 293T cells we observed that the Sts-1 RH/AA mutant did not affect EGFR phosphorylation (Fig. 2B), while the corresponding mutations in Sts-2 (Sts-2 RH/AA) did not affect receptor phosphorylation (not shown). These data are consistent with a recent report of Mikhailik et al. who solved the crystal structure of the Sts-1 PGM domain and showed that it possesses phosphatase activity which was required for the ability of Sts-1 to regulate TCR signaling in T-cells [12]. On contrary, Sts-2-though containing all catalytically active residues – showed only marginal phosphatase activity in vitro, when compared to the activity of Sts-1 [12]. Interestingly, the active site of Sts-1/2 requires HHRR residues, which is the characteristic two-step catalytic mechanism of phosphoglyserate mutase/acid phosphatase family enzymes. Indeed, prostatic acid phosphatases were shown to dephosphorylate a wide range of phosphorylated substrates including ATP, ADP pyrophosphate and large tyrosine phosphorylated peptides including proteins such as the ErbB2 receptor [11,13,14]. However, the catalytic mechanism of PGM enzymes appears to be different from the prototypical tyrosine phosphatase, which have cysteine-based catalytic sites [3].

Classical protein-tyrosine phosphatases exhibit a high degree of substrate specificity and often dephosphorylate only a subset of phosphorylated tyrosins present on proteins [15,16]. This site-selectivity allows a modulation of the downstream signalling events. We wanted to know whether Sts-1 preferentially acts on specific tyrosines or unspecifically dephosphorylates the EGFR. Therefore, we overexpressed Sts-1 or Sts-1 RH/AA in Hela cells and analyzed the phosphorylation pattern of endogenous EGFR using antibodies specific for individual phosphorylated tyrosines in the cytoplasmic region of EGFR. We found that Sts-1 wild type but not Sts-1 RH/AA potently dephosphorylated all tested tyrosines including Y845, Y992 (PLCγ-binding site), Y1045 (Cbl-binding site), Y1068 (Grb2 and Gab1) and Y1173 (PLCγ and Shc) (Fig. 2B). Sts-2 in contrast, did not affect the phosphorylation of any tested tyrosine (data not shown). We next wanted to confirm that the effect of Sts-1 on EGFR is direct or mediated by another protein. We therefore purified phosphorylated EGFR from EGF-treated

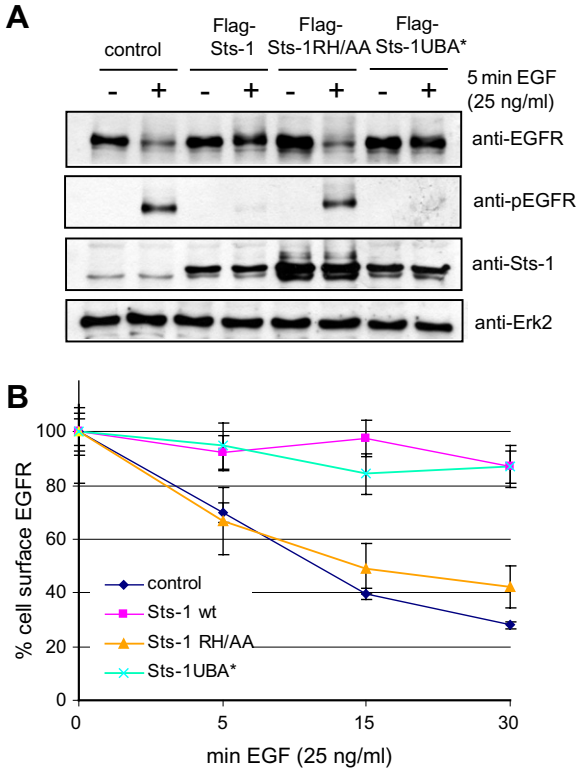

Fig. 3. The phosphatase activity of Sts-1 is required for its ability to inhibit EGFR degradation and internalization. (A) Overexpression of Sts-1 UBA* has the same stabilizing effect on EGFR as the Sts-1 wild type. In contrast Sts-1 RH/AA is unable to inhibit receptor degradation. Hela cells transfected with the indicated constructs were starved and stimulated with 25 ng/ml EGF for 5 min or left unstimulated. Total cell lysates were analyzed by SDS–PAGE and immunoblotting. (B) Internalization of endogenous EGFR was monitored in HeLa cells transfected with control vector, Sts-1 wild type, Sts-1 RH/AA or Sts-1 UBA*. Sts-1 wild type and the Ub-binding deficient Sts-1 UBA* stabilized EGFR at the cell surface whereas Sts-1 RH/AA expressing cells internalized the receptor with a similar efficiency as the control cells. EGFR residing at the cell surface after 0, 5, 15 and 30 min of EGF stimulation (25 ng/ml) was quantified flow cytometry using a PE-coupled EGFR antibody. The expression levels of transfected proteins were checked by immunoblotting. The data represent the means ± S.E.M. of three experiments.

HeLa cells by immunoprecipitation and incubated it in vitro with Sts-1 or Sts-1 RH/AA that had been purified from starved HEK 293T-cells (Fig. 2C). Under these conditions Sts-1 potently dephosphorylated EGFR while Sts-1 RH/AA was completely inactive, suggesting that EGFR is a direct target of Sts-1.

Sts-2 was proposed to stabilize EGFR by interfering with the Ub-dependent transport machinery and its ability to bind to Ub via its N-terminal UBA domain was essential for blocking receptor downregulation [5,9]. Given the fact that Sts-1 caused dephosphorylation of the receptor and Sts-1 RH/AA significantly affected EGFR levels we wondered about the role of the Sts-1 UBA-domain in receptor stabilization. We first analyzed receptor degradation in the presence of Sts-1 wild type (Sts-1), Sts-1 phosphatase mutant (Sts-1 RH/AA) or Ub-binding deficient Sts-1 UBA mutant (Sts-1-UBA*) in HeLa cells (Fig. 3A). Overexpression of Sts-1 wild type and Sts-1 UBA* lead to stabilization of EGFR upon stimulation with 25 ng/ml EGF, whereas the phosphatase-inactive mutant Sts-1 RH/AA allowed a normal receptor degradation suggesting that the phosphatase activity of the PGM domain is principally responsible for the inhibition of receptor degradation. We next analyzed the contribution of UBA and PGM domains to the internalization of endogenous EGFR by flow cytometry. As shown in Fig. 3B, overexpression of Sts-1 wild type resulted in the stabilization of the receptor at the cell surface upon cell stimulation with 25 ng/ml EGF. Mutations in the UBA-domain of Sts-1 did not affect this ability significantly. In presence of the phosphatase-inactive mutant Sts-1 RH/AA the receptor was removed from the cell surface to a similar extent as in the control cells.

The results presented above suggest a model in which Sts-1 blocks EGFR signalling, internalization and degradation by dephosphorylating the receptor thereby removing the docking sites for the downstream effectors. This model was further corroborated by the observation that Sts-1 overexpression lead to the loss of Cbl from EGFR complexes (Fig. 4A). Interestingly, at the same time Sts-1 was still present in the complexes indicating that once recruited to the receptor Sts-1 can stay associated with the receptor independently of Cbl. Though this is in agreement with the ability of Sts-1 to dephosphorylate activated EGFR in the absence of Cbl in vitro (Fig. 2C), to date we do not have further experimental evidence for a direct binding or Cbl-independent way of association between Sts-1 and EGFR under physiological conditions.

Lastly, we studied whether Cbl, which is phosphorylated upon EGF or T-cell receptor stimulation [17,18], is dephosphorylated by Sts-1. Cbl-phosphorylation can be mediated by Src, Fyn, Yes and Syk kinases and several RTKs depending on the cellular context [19] [18] and was shown to be required for its function as Ub E3-ligase and adaptor protein [18,20]. Overexpression of Sts-1, but not Sts-1 RH/AA, in cells led to potent dephosphorylation of endogenous Cbl (Fig. 4B). To avoid the influence of EGFR dephosphorylation on the Cbl-phosphorylation status we purified phosphorylated Cbl from EGF-stimulated Hela cells and incubated it with Sts-1 or Sts-1 RH/AA in the same way as described above with EGFR. As shown in Fig. 4C, in addition to EGFR, Sts-1 dephosphorylates and thus inactivates Cbl. In this way Sts-1 recruitment results in termination of both Ub-dependent and -independent internalization pathways. It remains to be determined to which extent EGFR signals are emitted before the Cbl/Sts-1 complex

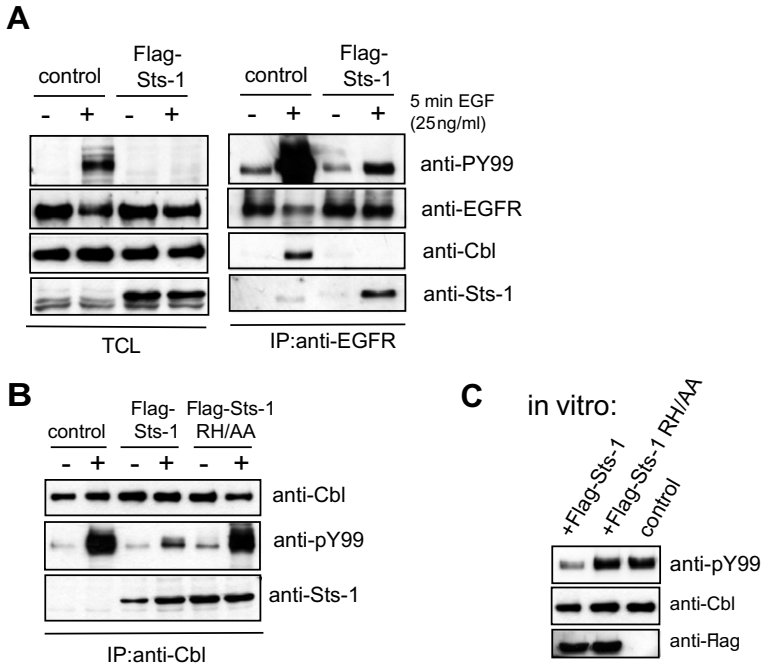

Fig. 4. Sts-1 phosphatase activity prevents Cbl-mediated EGFR downregulation. (A) Overexpression of Sts-1 leads to loss of Cbl from EGFR complexes. HeLa cell lysates expressing the indicated constructs were subjected to immunoprecipitation using an anti-EGFR antibody and analyzed by SDS–PAGE and immunoblotting. (B) Sts-1 overexpression suppresses EGF-induced phosphorylation of endogenous Cbl. HeLa lysates expressing the indicated constructs were subjected to immunoprecipitation using anti-Cbl (RF) antibody and analyzed by SDS–PAGE and immunoblotting. (C) Cbl is a direct target of Sts-1 phosphatase activity. Phosphorylated Cbl was purified from EGF treated HEK 293T-cells by immunoprecipitation and incubated for 30 min at 37 °C with FLAG-Sts-1 wild type or FLAG-Sts-1 RH/AA purified from cell lysates overpressing the proteins.

reaches the activated receptor upon overexpression of Sts-1. However, at endogeous levels it seems that Sts-1 is present in EGFR-positive endosom populations indicating that it may act as a negative feed-back mechanism which may be involved in slowing down endocytic processes and/or inhibit signalling competence of the EGFR associated complexes.

Taken together, this report describes an unexpected difference between the two related proteins Sts-1 and Sts-2. Upon cell stimulation both proteins are recruited into activated EGFR receptor complexes via the E3 ligase Cbl and potently inhibit receptor endocytosis upon overexpression. However, whereas Sts-2 requires a functional UBA domain to achieve receptor stabilization, Sts-1 does so by dephosphorylating the receptor as well as the negative regulator Cbl. In this way Sts-1 not only terminates receptor downregulation but also receptor signalling. As a consequence Sts-1-containing receptor complexes are inactive whereas Sts-2-containing complexes are signalling competent. In accordance, we have observed that overexpression of Sts-2, but not Sts-1, leads to oncogenic transformation in cultured fibroblasts [5]. This functional difference in Sts-1 and Sts-2 action might also explain previous observations in T-cells whereby Sts-2 positively regulated TCR-dependent gene transcription [6], while Sts-1 acted as a suppressor of TCR signalling [7]. Yet, Sts-1 and Sts-2 were shown cooperate to negatively regulate T-cell receptor signalling in vivo since only upon deletion of both Sts-1 and Sts-2 there was a detectable phenotype observed, which included hyperphosphorylation of Zap-70 and increase proliferation of T-cells [7]. Future studies on the interplay between Sts-1 and Sts-2 as well as the importance of the different domains of Sts-1 and Sts-2 will be essential to delineate their distinct biological functions in cells.

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